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R-roscovitine (CYC202) alleviates renal cell proliferation in nephritis without aggravating podocyte injury

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R-roscovitine (CYC202) alleviates renal cell proliferation in nephritis without aggravating podocyte injury.

Background. Cyclin-dependent kinase (CDK) inhibition is a new therapeutic approach to proliferative glomerulonephritides. CDK2 is required for G_1/S transition and DNA synthesis and is inhibited by CYC202 (R-roscovitine). Since podocytes express CDK2 in nephritis and since loss of podocytes contributes to glomerulosclerosis, the rationale of the present study was to test whether CDK2 inhibition is safe in instances of podocyte injury.

Methods. Rats with passive Heymann nephritis, a model of membranous glomerulonephritis, were treated (day 3 to 30) with vehicle, low (25 mg/kg/day), or high (50 mg/kg/day) doses of CYC202.

Results. On day 27, blood pressure was normal in nephritic controls and was dose-dependently reduced by CYC202. Urinary albumin excretion did not differ between the groups on days 9, 16, 23, and 30. To investigate podocyte injury, we assessed the glomerular de novo expression of desmin, which was markedly up-regulated in almost all passive Heymann nephritis glomeruli but was not significantly different between the three groups. No tubulointerstitial de novo expression of desmin or alpha-smooth muscle actin (α-SMA), or tubulointerstitial monocyte/macrophage infiltration was noted in any group. Biologic activity of CYC202 was evident in the form of a dose-dependent decrease in the number of glomerular and tubulointerstitial mitotic figures as compared to vehicle alone. Glomerular immunostaining for cyclin D1, a marker for G_0 to G_1 transition, was significantly decreased in CYC202 treated groups at day 9.

Conclusion. Whereas inhibition of CDKs by CYC202 reduced intrarenal cell proliferation in passive Heymann nephritis it did not aggravate podocyte damage, suggesting that this novel therapeutic approach is safe in renal diseases characterized by podocyte injury.

[1]Both authors contributed equally to this work.

Key words: CDK2, membranous nephropathy, cell cycle, podocyte, proteinuria.

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Glomerulonephritides and systemic immunologic diseases account for 20% to 25% of terminal renal failures in most Western countries. Many progressive immune-mediated glomerular diseases are characterized by mesangial proliferative changes and would thus potentially benefit from therapy with a cell cycle inhibitor. However, all of these glomerular diseases, as well as metabolic glomerular diseases such as diabetic nephropathy, appear to advance to glomerular scaring through secondary podocyte damage [1–3]. Other progressive glomerulonephritides do not exhibit mesangial proliferative changes but rather primary podocyte injury. A classic disease of this group is membranous glomerulonephritis, where an (auto) immune process leads to podocyte damage without significant changes inside the glomerular tuft. Primary or secondary podocyte damage determines the rate of glomerular and subsequent renal tubulointerstitial scaring [3].

The particular role of the podocyte as a final common pathway of glomerular scaring has traditionally been explained by the terminal differentiation of these cells and the assumption that a damaged podocyte can not be replaced, resulting in areas of bare glomerular basement membrane (GBM), which then fuse with the outer Bowman's capsule and thereby generate a first adhesion between glomerular tuft and capsule. Such adhesions are viewed as the starting point of segmental and ultimately global glomerulosclerosis [3, 4].

Recently, the view of the podocyte as a terminally differentiated cell has been challenged. Observations of rare, yet definitive podocyte DNA synthesis and mitoses in glomerulonephritis, and bi- (multi-) nucleation rather than increased cell numbers, suggest that injured podocytes in principle possess the ability to replicate but instead engage in a defective cell cycle with acytokinetic mitosis [5]. Normal and/or injured podocytes contain high levels of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 [6, 7], which would prevent cell cycle progression. However, they have also been demonstrated to express CDK2, a promoter of cell cycle progression,

during passive Heymann nephritis [7], a model of human membranous glomerulonephritis in rats [8]. This model is therefore of particular use to study the effects of CDK2 inhibition in podocyte disease, given the concern that CDK2 inhibition might impair the little adaptive response that podocytes can exhibit and thereby aggravate the development of glomerulosclerosis. However, CDK2 inhibition in passive Heymann nephritis might also be beneficial. Very recent data show that engagement of podocytes in the cell cycle may alter their adhesive properties in such a way that they are lost in the urine [with all the potential consequences described above (i.e., denudation of basement membrane and adhesion formation)] [9]. In this scenario, it might even be beneficial to prevent the podocyte from engaging in the cell cycle. The aim of our study was therefore to determine the effects of currently employed therapeutic doses of a CDK2 inhibitor, R-roscovitine (CYC202), in passive Heymann nephritis. CYC202, a purine analogue, inhibits the activity of CDK2 but also of other CDKs, including CDK1 (cdc2), CDK5, CDK7, and CDK9, by binding to their adenosine triphosphate (ATP) binding pocket [10]. However, the affinity of CYC202 to the different CDKs is variable with inhibition constants $(IC)_{50}$ of 0.1 μmol/L for CDK2/cyclin E, 0.7 μmol/L for CDK2/cyclin A, 0.5 μmol/L for CDK7/cyclin H, 0.84 μmol/L for CDK9/cyclin T1, and 2.7 μmol/L for CDK1/cyclin B [11].

METHODS

Experimental design

Male rats (Sprague-Dawley, Charles River Wiga GmbH, Sulzfeld, Germany), weighing 180 to 210 g at the start of the experiment, were used in the experiments. Rats were housed in cages under conditions of constant temperature (22°C) and humidity (50%), with a 12-hour dark/light cycle. The animals had free access to tap water and standard rat chow. Passive Heymann nephritis was induced on day 0 by intravenous injection of 0.3 mL of sheep anti-Fx1A antibody per rat, prepared as described previously [8]. All animal experiments were approved by the local review boards.

Treatment with CYC202 was started on day 3, in order to avoid interference with the induction phase of passive Heymann nephritis, and continued until day 30. Treatment consisted of once-daily oral gavage of CYC202 until day 10. CYC202 was dissolved in 30 mmol/L HCl and stirred with a magnetic stirrer until dissolution. After day 10, treatment was switched to daily intraperitoneal injections. The vehicle used here was dimethyl sulfoxide (DMSO) (ICN Biomedicals, Aurora, OH, USA). Three groups of rats were studied: group I, nephritic untreated group ($N = 10$) (animals received vehicle alone); group II, nephritic low dose treatment group ($N = 10$) (animals were treated once daily with 25 mg CYC202/kg/day); and group III, nephritic high dose treatment group ($N = 10$) (animals were treated once daily with 50 mg CYC202/kg/day).

To determine urinary albumin excretion, 24-hour urine collections were performed in metabolic cages on days 9, 16, 23, and 30 after disease induction.

Renal biopsies for histologic evaluation were obtained on day 9 by intravital biopsy and during postmortem on day 30 after disease induction. The thymidine analogue 5-bromo-2′deoxyuridine (BrdU) (100 mg/kg body weight) (Sigma Chemical Co., St. Louis, MO, USA) was injected intraperitoneally at 4 hours before sacrifice on day 30. All rats were sacrificed under isoflurane anesthesia and blood was collected by puncture of the vena cava inferior 5 hours after administering the last dose of CYC202. Kidneys were harvested and prepared for histologic examination.

Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [12] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin.

Using a 400-fold magnification of the PAS-stained sections, the total number of mitoses within the glomerular tuft (extrapolated to mitoses per 100 glomerular cross sections) was counted. To determine this, between 20 and 100 glomerular profiles were evaluated per specimen on day 9 and between 100 and 190 glomerular profiles were evaluated on day 30.

In addition, the number of mitoses in the tubulointerstitium was evaluated using a grid composed of 100 fields at a magnification of 400-fold, so that every field corresponded to an area of 0.0625 mm^2. One hundred grid fields in the renal cortical tubulointerstitium were analyzed and mean counts per kidney were obtained.

Electron microscopy was performed following standard protocols [13]. Blocks of renal tissue (1 mm^3) were fixed in a solution of 2% formaldehyde and 2.5% glutaraldehyde according to Karnovsky's method, with cacodylate buffer (0.2 mol/L, pH 7.4). After fixation, the samples were dehydrated and embedded in epoxy resin (glycide ether 100) (Serva, Heidelberg, Germany). Sections were cut and stained with toluidine-blue for light microscopy prescreening. Ultrathin sections were then cut at 80 to 100 nm, stained with uranyl acetate and lead citrate, and viewed and photographed in a Philips TEM 400 transmission electron microscope. For evaluation of slit numbers per length of GBM, images were obtained from random areas, enlarged 13,000-fold and the number of filtration-slits per GBM length was measured.

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by an indirect immunoperoxidase technique [12]. Primary antibodies included [14]: (*1*) a monoclonal IgG1 antibody (clone D33) against human muscle desmin (1:100) (Dako, Glostrup, Denmark); (*2*) cyclin D1, a rabbit polyclonal antibody (bcl-1 Ab-3) (1:100) (Lab Vision, Fremont, CA, USA) raised against the C-terminal portion of cyclin D1 of rat origin [15]; (*3*) ED1 (1:500)(Serotec, Oxford, UK), a monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic rat cells; (*4*) a monoclonal IgG antibody (clone 1A4) to an NH_2-terminal synthetic decapeptide of human alpha-smooth muscle actin (α-SMA) (1:500) (Dako); (*5*) BU-1, a monoclonal antibody against bromo-deoxyuridine (Amersham, Braunschweig, Germany); (6) proliferating cell nuclear antigen (PCNA) (Ab-1) (clone PC10) (1:1000) (Oncogene Science Inc., Uniondale, NY, USA) a mouse monoclonal IgG antibody that reacts with the human PCNA, which is expressed in a cell cycle dependent manner [16]; and (*7*) WT1 (F-6) (1:300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal IgG1 that reacts with the WT1 antigen on podocytes and epithelial cells of the Bowman capsule.

Desmin staining was evaluated using a point counting method. For this, a grid composed of 121 dots was superimposed on at least 20 glomeruli (magnification 600-fold) and the percentage of dots overlying stained areas were counted. Similarly, tubulointerstitial α-SMA staining was evaluated by superimposing the grid on at least 50 tubulointerstitial grid fields measuring 0.027 mm^2 each.

The glomerular expression of cyclin D1 was analyzed on days 9 and 30. Thirty glomerular cross sections were evaluated per sample and the results were expressed as the mean number of nuclei/cells staining positively per 30 glomerular cross sections. To obtain total counts of infiltrating monocytes/macrophages in the renal cortical tubulointerstitium, 100 grid fields (see above) were analyzed and mean counts per kidney were obtained.

To obtain mean numbers of proliferating (PCNA+) cells in glomeruli on day 9 at least 20 consecutive cross sections of glomeruli (magnification 600-fold) were evaluated and mean values per glomerular cross sections were calculated. In addition, we determined the number of PCNA+ cells at the edge of the glomerular tuft, presumed to represent PCNA+ podocytes.

To further evaluate whether these cells represented proliferating podocytes, we performed double stainings for PCNA and the podocyte specific marker WT1 on day 30. First, sections were stained for WT1 using a blue alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA, USA) generating a blue color product. On the next day, proliferating cells were stained with our PCNA antibody using an immunoperoxidase procedure, including 3-amino-9-ethylcarbazole (AEC) generating a red color product. Cells were identified as proliferating podocytes if they showed both positive nuclear stainings for PCNA and WT1, generating a blueish-purple color product.

Negative controls for all of the immunohistochemical analyses included substitution of the primary antibody with normal IgG from the same species as the primary antibody at a similar concentration. Evaluation of all slides was performed by an investigator who was unaware of the origin of the slides.

Miscellaneous measurements

Blood pressure was measured by tail-cuff plethysmography on day 27 in conscious rats using a programmed sphygmomanometer (BP-981) (Softron, Tokyo, Japan). For each animal, at least three consecutive measurements were performed.

CYC202 serum levels were determined on day 30 by mass spectroscopy (MS) (Quattro Ultima, Waters/Micromass UK Ltd., Manchester, UK) with serum extraction carried out following total protein precipitation. CYC202 serum levels were determined from a standard calibration line over the linear range 0.01 μmol/L to 7.2 μmol/L with appropriate sample dilutions with control rat serum (Wistar, mixed gender) (Harlan Seralab UK, Ltd., Loughborough, UK) made where necessary.

Creatinine was measured in serum using an autoanalyzer (Beckman Instruments GmbH, München, Germany).

Urinary albumin excretion was determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) (Nephrat) (Exocell Inc., Philadelphia, PA, USA).

Statistical analysis

All values are expressed as mean ± SD or as individual data. Statistical significance (defined as $P < 0.05$) was evaluated using one-way analysis of variance (ANOVA) with modified *t* test performed with the Bonferroni correction [17].

RESULTS

Animal behavior, body weight, and CYC202 serum levels

Four rats died (1 to 2 in each group) during the first 10 days of the experiment due to technical failure. All other rats were active and appeared healthy, and did not develop evidence of peritonitis, ascites, or diarrhea during the study.

Prior to disease induction, there were no significant differences between the three groups in terms of body weight. Rats from all groups gained weight in the next 30 days, although the body weights of the vehicle-only–treated rats were moderately higher than those of low-dose or high-dose CYC202-treated rats at each time point

Table 1. Body weights, CYC202 serum levels, serum creatinine, and blood pressures of the experimental groups following the induction of passive Heymann's nephritis

Body weight g	Nephritic + vehicle ($N = 9$)	Nephritic + 25 mg/kg/day CYC202 ($N = 9$)	Nephritic + 50 mg/kg/day CYC202 ($N = 8$)
Day 7	202 ± 9	199 ± 11	197 ± 11
Day 14	255 ± 15	243 ± 21	225 ± 17
Day 21	288 ± 18	274 ± 23	249 ± 18
Day 28	304 ± 15	290 ± 22	270 ± 13[a]
CYC202 serum concentration (day 30) *μmol/L*	<0.01	5 ± 2	15 ± 8
Serum creatinine (day 30) *μmol/L*	35 ± 5	35 ± 8	45 ± 10[a,b]
Mean blood pressure (day 27) *mm Hg*	92 ± 6	85 ± 6[a]	76 ± 4[a]

[a] $P < 0.05$ versus group receiving vehicle; [b] $P < 0.05$ versus group receiving 25 mg/kg/day CYC202.

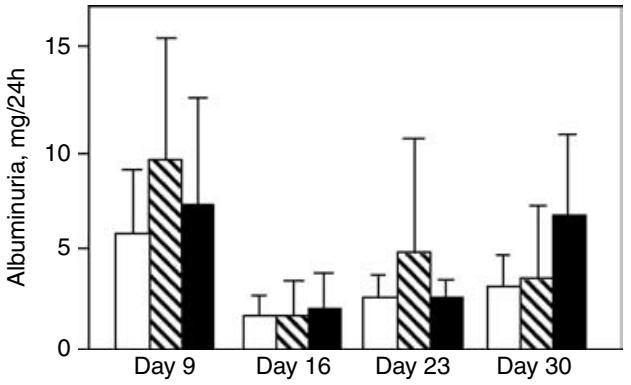

Fig. 1. Urinary albumin excretion in rats with passive Heymann nephritis on days 9, 16, 23, and 30 receiving either vehicle or CYC202 from day 3 to 30 after disease induction. The normal albuminuria determined in five healthy rats of the same age, gender, and weight was 0.11 ± 0.04 mg/24 hours. Rats receiving vehicle-only (□) ($N = 9$); rats receiving 25 mg/kg/day of CYC202 (▨) ($N = 9$); rats receiving 50 mg/kg/day of CYC202 (■) ($N = 8$).

(Table 1). At day 30, the body weight of the high-dose CYC202-treated group was significantly lower as compared to the vehicle-only–treated group.

Determination of CYC202 serum levels on day 30 revealed a mean level of 4.9 μmol/L in the rats receiving 25 mg/kg/day and threefold higher levels in the group receiving 50 mg/kg/day (Table 1).

Renal function, proteinuria, and blood pressure

Serum creatinine values remained within the normal range in vehicle-treated and low-dose CYC202 rats. In the high-dose CYC202-treated group serum creatinine was significantly increased as compared to the other groups on day 30, although it still remained in the normal range (Table 1).

As shown in Figure 1, urinary albumin excretion peaked on day 9, when it was about 70-fold elevated over the normal range, and then decreased until day 16 in all three groups. From day 16 until the end of the study urinary albumin excretion remained 30-fold elevated over controls. No statistically significant differences between the groups were noted at any of the time points measured.

Mean systolic blood pressures in nephritic rats receiving vehicle were within the normal range on day 27 (Table 1). Treatment of nephritic rats with CYC202 resulted in a dose-dependent decrease of mean systolic blood pressure (Table 1).

Morphologic studies

Glomerular changes. Examination of PAS-stained sections obtained from kidneys on days 9 and 30 revealed no evidence for focal or global glomerulosclerosis in any specimen (Fig. 2A).

Ultrastructural examinations of kidney specimens obtained at day 30 following passive Heymann's nephritis induction revealed typical changes as previously reported in passive Heymann's nephritis. These changes included frequent electron-dense deposits within the basement membrane and the subepithelial space, segmental flattening and fusion of podocyte foot processes, as well as hypertrophy of the podocyte cell body (Fig. 2B). For quantification of podocyte damage, the number of filtration slits per glomerular basal membrane length was evaluated in three high-dose CYC202-treated rats (26 randomly chosen areas, 319 slits) and three control animals (21 randomly chosen areas, 245 slits). In untreated rats, 1.19 ± 0.2 slits were counted per micrometer of GBM vs. 1.07 ± 0.07 per micrometer GBM in high-dose CYC202 animals (P = NS) (data not shown). In summary, we were unable to identify any ultrastructural differences between rats that had received control compound, low doses, or high doses of CYC202/roscovitine regarding both the incidence as well as the severity of glomerular lesions.

To specifically investigate glomerular injury in the rats with passive Heymann's nephritis, we assessed the glomerular de novo expression of desmin, which in passive Heymann's nephritis can serve as a specific marker of podocyte injury [18]. As expected, desmin expression was markedly up-regulated in almost all glomeruli on days 9 and 30 (Fig. 3A and B), confirming earlier observations [18]. However, we did not detect any significant difference between the CYC202-treated groups and the animals treated with vehicle-only at either time point (Fig. 3C). Of note, if at all, desmin expression tended to be lower on day 30 in the nephritic groups receiving CYC202. Glomerular de novo expression of α-SMA, a marker of activated mesangial cells [19], was not observed in any specimen (data not shown).

To assess the biologic activity of CYC202 in vivo in addition to measuring serum levels (see above), we next

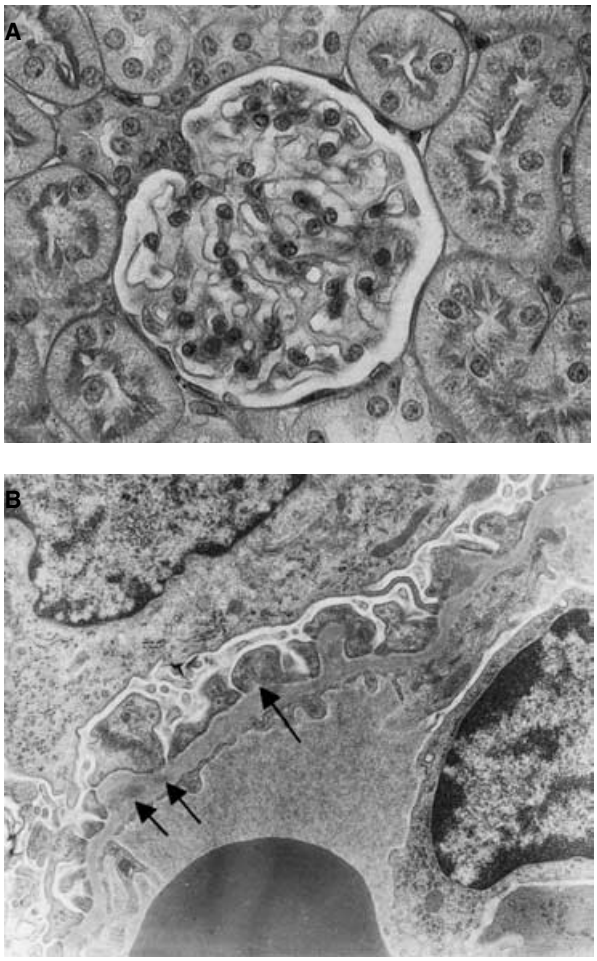

Fig. 2. Morphologic changes in rats with passive Heymann's nephritis on day 30 treated with high-dose CYC202. (*A*) Periodic acid-Schiff (PAS) staining. Regular glomerular structure without signs of glomerulosclerosis (magnification ×600). (*B*) Transmission electron microscopy. Ultrastructural changes included widespread electron-dense deposits within the basement membrane and the subepithelial space (arrows), irregular basement membrane protrusions, as well as flattening and fusion of podocyte foot processes (magnification ×13,000). Similar observations were made in rats with low-dose CYC202 or vehicle.

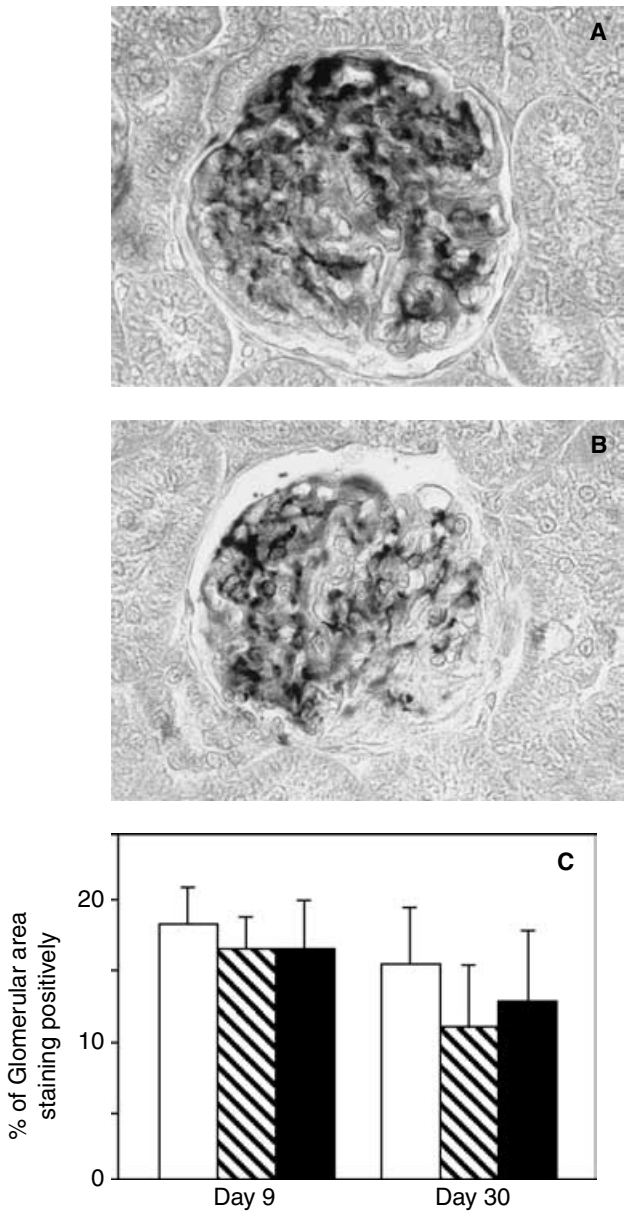

Fig. 3. Glomerular expression of desmin. (*A*) In vehicle-treated nephritic rats on day 30, desmin expression in glomeruli is widespread and mostly locates to podocytes (magnification ×400). (*B*) In nephritic rats receiving high-dose CYC202, the expression of desmin on day 30 is similar (magnification ×400). (*C*) Quantitative assessment of glomerular desmin expression in rats with passive Heymann's nephritis receiving either vehicle or CYC202 from day 3 to 30 after disease induction. All differences were not significant. Rats receiving vehicle only (□) ($N = 9$); rats receiving 25 mg/kg/day of CYC202 (▨) ($N = 9$); rats receiving 50 mg/kg/day of CYC202 (■) ($N = 8$).

investigated cell proliferation and the expression of cyclin D1.

Compared to the vehicle-only group, treatment with low-dose CYC202 decreased the number of glomerular mitotic figures at day 30 by 22% and by 61% in the high-dose group (Fig. 4), a statistically significant reduction. Cell proliferation appeared to be equally reduced in both mesangial and glomerular endothelial cells in CYC202-treated animals as compared to the vehicle group, judged by the location of mitotic figures in PAS-stained sections (data not shown). Cell proliferation was also assessed by counting of BrdU-positive glomerular cells. It was significantly lower in the high-dose CYC202-treated group when compared to the vehicle-only–treated group (3.3 ± 2.4 vs. 19.2 ± 8.3 BrdU-positive nuclei/100 glomeruli in the high-dose vs. vehicle group, respectively) ($P <$ 0.05). Reduced glomerular cell proliferation in CYC202-treated animals was not due to changes in glomerular monocyte/macrophages, the counts of which remained in the normal range in all specimens (data not shown).

Since mitoses and BrdU incorporation in cells at the edge of the glomerular tuft (i.e., podocytes), occurred too infrequently on day 9 of passive Heymann's nephritis

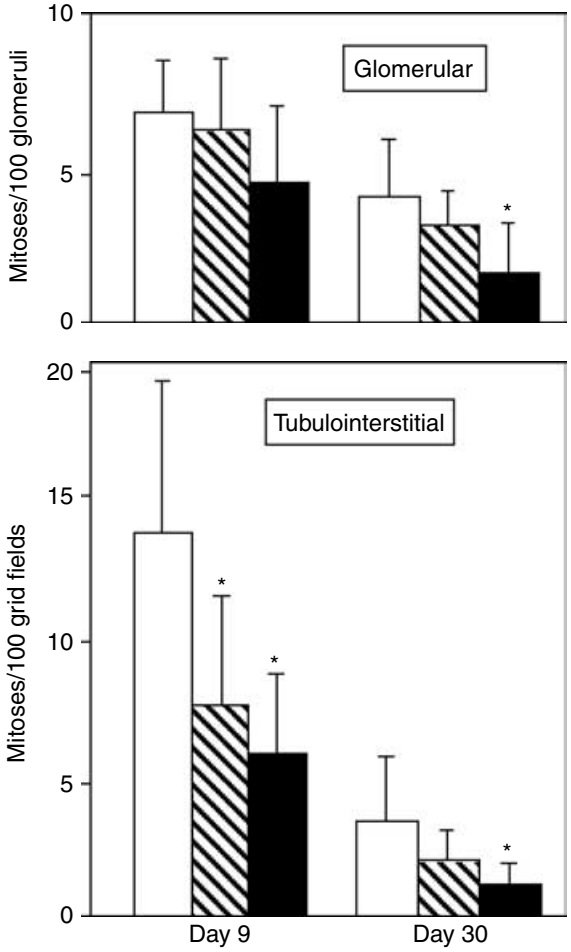

Fig. 4. Quantitative evaluation of cell proliferation (as defined by counts of mitotic figures) in glomeruli and the tubulointerstitium of rats with passive Heymann's nephritis receiving either vehicle or CYC202 from day 3 to 30 after disease induction. $^*P < 0.05$ versus nephritic rats receiving vehicle alone. Rats receiving vehicle only (□) ($N = 9$); rats receiving 25 mg/kg/day of CYC202 (▨) ($N = 9$); rats receiving 50 mg/kg/day of CYC202 (■) ($N = 8$).

to be quantified, we also stained the sections for PCNA. The number of PCNA+ cells per glomerular cross section and, more important, at the edge of the glomerular tuft decreased significantly on day 9 in both low- and high-dose CYC202-treated groups (Table 2).

Additional double stainings for the podocyte-specific WT1 and PCNA on day 30 showed no significant difference in low-dose or high-dose–treated animals when compared to untreated animals (0.38 ± 0.3 vs. 0.52 ± 0.4 positive cells/glomerulus in the low-dose group compared to untreated animals and 0.9 ± 0.6 vs. 0.52 ± 0.4 positive cells/glomerulus in the high-dose animals when compared to untreated animals).

Cyclin D1 expression, a marker of cell entry into the G_1 phase of the cell cycle, was observed in some glomerular cells in a cytoplasmic or nuclear location (Fig. 5A and B). This is consistent with observations in normal rat kidney in vitro, where cyclin D1 localized to the cytoplasm and was shifted to the nucleus during cell proliferation [20]. The glomerular expression of cyclin D1–positive cells decreased significantly on day 9 in both groups receiving CYC202. This difference did not persist on day 30 (Fig. 5C), suggesting that at this stage CYC202 no longer prevented entry of cells into the cell cycle, yet still interfered with completion of the cell cycle, as shown by persistently reduced mitosis rates and PCNA positivity (Fig. 4) (Table 2).

Table 2. Proliferating cell nuclear antigen positive (PCNA+) cells on day 9

Group	Nephritic + vehicle ($N = 9$)	Nephritic + 25 mg/kg/day CYC202 ($N = 9$)	Nephritic + 50 mg/kg/day CYC202 ($N = 8$)
Number of PCNA+ cells/glomerular cross section	6.7 ± 1.8	3.9 ± 1.2[a]	3.8 ± 0.8[a]
Number of PCNA+ cells at the edge of the glomerular tuft/ glomerular cross section	2.3 ± 0.7	1.1 ± 0.4[a]	0.8 ± 0.5[a]

[a] $P < 0.05$ versus group receiving vehicle.

Tubulointerstial changes. No tubulointerstitial damage occurred in any group of rats as judged by PAS staining of the sections and by the absence of de novo interstitial α-SMA and desmin expression (data not shown) (i.e., two markers of myofibroblast transformation or epithelial transdifferentiation [21]).

Further evidence of a biologic effect of CYC202 was obtained by assessing tubulointerstitial mitosis rates. In comparison to vehicle-treated nephritic rats, the number of mitotic figures in the tubulointerstitium decreased in a statistically significant manner with low-dose CYC202 treatment at day 9 and with high-dose CYC202 treatment at days 9 and 30 (Fig. 4). Similar data were obtained, when mitotic rates were determined within the tubular cells only (data not shown). Reduced tubulointerstitial cell proliferation in low-dose and high-dose CYC202-treated rats was also confirmed by quantification of BrdU-positive nuclei (high dose 32 ± 7 BrdU-positive nuclei per 100 grids; low dose 52 ± 20; vehicle 84 ± 34) ($P < 0.05$). Tubulointerstitial monocyte/macrophage counts remained unaffected by CYC202 treatment on day 9, but were significantly lower in the high-dose CYC202 group as compared to vehicle-treated rats on day 30 (Fig. 6).

DISCUSSION

CDKs are key regulators of the cell cycle, which become active when they associate with their respective cyclin subunits, proteins that are present only at specific stages of the cell cycle [22, 23]. Of the CDKs inhibited by

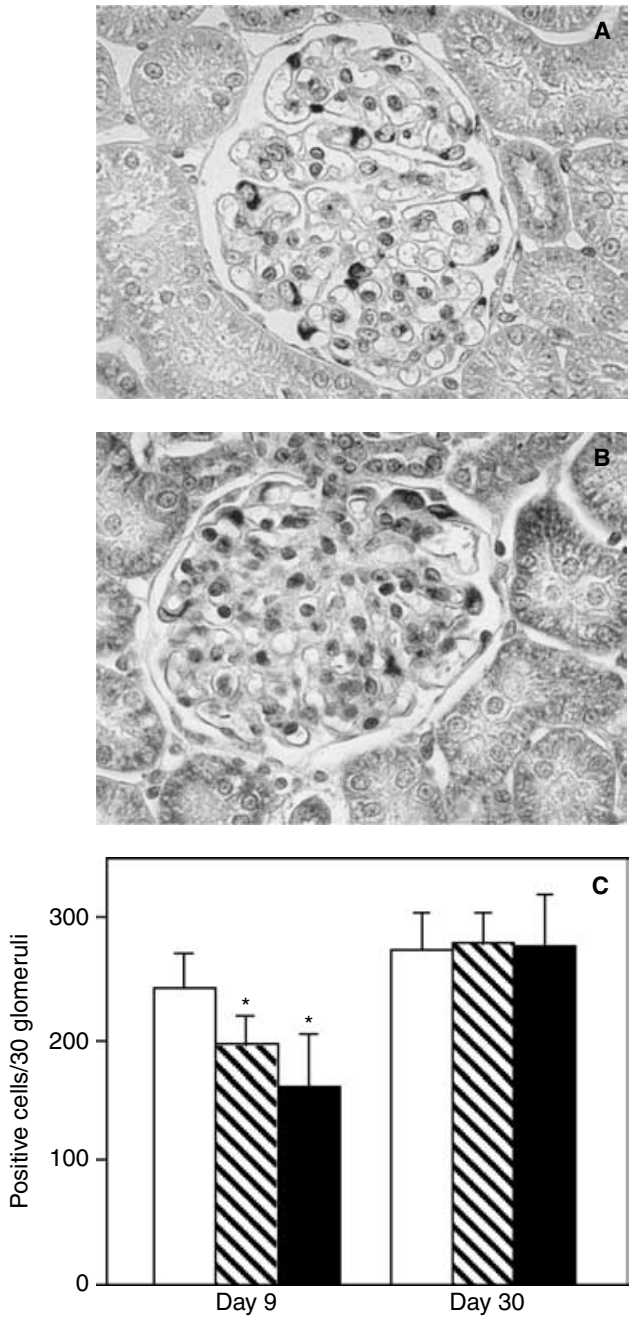

Fig. 5. Immunostaining for cyclin D1 in rats with passive Heymann's nephritis receiving either vehicle or CYC202 from day 3 to 30 after disease induction. (*A*) In vehicle-treated nephritic rats on day 9, cyclin D1 is expressed mostly in the cytoplasm (magnification ×400). (*B*) In nephritic rats receiving high-dose CYC202, the expression of cyclin D1 on day 9 is qualitatively similar as in rats receiving vehicle, although quantitatively reduced (magnification ×400). (*C*) Quantitative assessment of glomerular cells staining positive for cyclin D1. $^*P < 0.05$ versus nephritic rats receiving vehicle alone. Rats receiving vehicle only (□) ($N = 9$); rats receiving 25 mg/kg/day of CYC202 (▨) ($N = 9$); rats receiving 50 mg/kg/day (■) of CYC202 ($N = 8$).

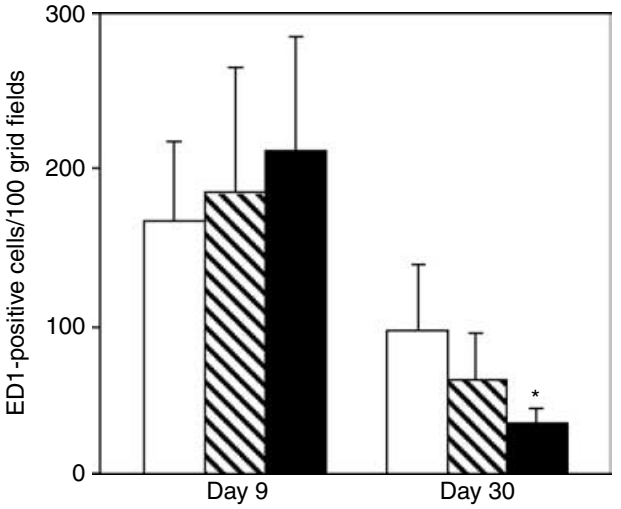

Fig. 6. Tubulointerstitial counts of monocytes/macrophages (ED1-positive cells) in rats with passive Heymann's nephritis receiving either vehicle or CYC202 from day 3 to 30 after disease induction. $^*P < 0.05$ versus nephritic rats receiving vehicle alone. Rats receiving vehicle only (□) ($N = 9$); rats receiving 25 mg/kg/day of CYC202 (▨) ($N = 9$); rats receiving 50 mg/kg/day of CYC202 (■) ($N = 8$).

CYC202 [11], CDK2 is clearly expressed and regulated in passive Heymann's nephritis (see above) and its inhibition likely constituted the major effect of CYC202 in the present study. It is possible, but at the plasma levels achieved less likely, that CDK1 was also affected and contributed to the activity of CYC202. In cell culture CDK1 is expressed in podocytes and is down-regulated by stretching or complement-mediated damage of the cells [24, 25]. Very recent data also describe CDK1 overexpression in passive Heymann's nephritis [26]. Effects on CDK5 are unlikely to contribute to our observations, since CDK5 mostly acts in the central nervous system [27], although very recent data suggest that CDK5 may play a role in podocyte differentiation during embryogenesis [abstract; Hiromura K, *et al*, *J Am Soc Nephrol* 13:32A, 2002]. Finally, CYC202 is also known to inhibit CDK7 and CDK9, CDKs involved in the phosphorylation of RNA polymerase II during transcription. Although currently no data exist on the expression of these two CDKs in passive Heymann's nephritis, there is evidence that both are expressed in the kidney [28, 29]. A reduced transcription rate resulting from CYC202-mediated inhibition of CDK7 and/or CDK9 would correlate with the lowering of cyclin D1 levels observed in this study. The impact of CYC202 would thus depend on the stage of the cell cycle of a particular cell at the time of exposure to the drug.

In a model of mesangioproliferative glomerulonephritis in rats, where the normally low level of glomerular CDK2 expression is markedly up-regulated, reducing the activity of CDK2 with roscovitine was beneficial, in that it decreased glomerular hypercellularity and matrix protein accumulation [30]. In contrast to mesangioproliferative changes, which are frequent in human glomerular disease, very few diseases are characterized by podocyte proliferation. These include the cellular variant of focal segmental glomerulosclerosis (FSGS) [31], collapsing glomerulopathy [32], and human immunodeficiency

virus (HIV)-associated nephropathy [32]. In the large majority of podocyte-related diseases, such as classic FSGS, membranous nephropathy, and minimal change disease, podocytes do not proliferate [33] although they may overexpress cell cycle regulatory proteins, including CDK2, and as a result enter the cell cycle [7, 34]. As discussed previously, in such instances the consequences of CDK2 inhibition for the podocyte might range from detrimental to beneficial.

The major finding of the present study was that CDK2 inhibition did not aggravate the course of the disease in a model bearing close similarity to human membranous glomerulonephritis. As assessed by albuminuria and signs of podocyte damage, no augmentation was noted in rats receiving either low- or high-dose CYC202, despite the fact that podocytes overexpress CDK2 in passive Heymann's nephritis [7, 34]. Our study thereby resolves an important safety concern, since primary or secondary podocyte damage is widespread in progressive glomerular disease [1–3]. On the other hand, despite a theoretic possibility (see above), we did not obtain clear evidence for a beneficial action of CYC202 on the course of passive Heymann's nephritis as judged by proteinuria. In contrast, in a model with prominent podocyte proliferation (i.e., collapsing glomerulopathy in mice), high-dose CYC202 (75 mg/kg body weight for 20 days) significantly improved glomerular lesions as compared to the control group exhibiting progressive disease [35].

In our study we also observed a reduction of systemic blood pressure in nephritic rats receiving CYC202. Transiently reduced blood pressure has also been noted in healthy dogs receiving high dose (500 mg/m^2) CYC202 (Cyclacel Ltd., Dundee, UK, unpublished data). Whether such a potentially beneficial side effect of CYC202 also occurs at therapeutic dosages in humans is currently unknown. Under the particular circumstances of the present study the dose-dependent blood pressure reduction (i.e., hemodynamic mechanisms), likely accounted for the observation of a mildly increased serum creatinine on day 30 in the high-dose CYC202 group since no morphologic evidence for irreversible renal damage was present. However, a possible toxicity of the drug cannot be ruled out with certainty.

Although we did not determine glomerular CDK2 activity directly, a number of observations suggest that CYC202 was active in vivo. First, serum levels in the present study obtained at 5 hours after the last CYC202 dose equated to 4.6 and 14.8 μmol/L, respectively, which corresponds to levels of the drug required for biologic activity (5 to 20 μmol/L). Second, CYC202 reduced both glomerular and tubulointerstitial cell proliferation (however, without leading to dectable pathology through this effect at least over the time course of the experiment). Third, we assessed expression of cyclin D1, which initiates cell cycle entry in early G_1 phase [22]. Increased expression of cyclin D1 has been described previously in rats with passive Heymann's nephritis [7] and was confirmed in the present study. More important, CYC202, at least transiently, decreased glomerular cyclin D1 expression in a significant manner. As discussed above, the reduction of cyclin D1 in CYC202-treated rats may be explained by effects of CYC202 on the "transcriptional CDKs" (i.e., CDK7 and CDK9). Taken together, these observations demonstrate that both low and high-dose CYC202 reduced renal cell proliferation likely through interference with CDK2 and possibly CDK7/CDK9. Finally, tubulointerstitial monocyte and macropahge counts were reduced by high-dose CYC202, indicating an effect on inflammatory cell responses. This phenomenon has also been observed with CYC202 previously in rat models of nephrectomy (unpublished data) (Cyclacel Ltd.). Again, this effect of CYC202 may be of interest for the treatment of progressive renal disease, given that anti-inflammatory actions, for example of mycophenolate mofetil, contributed to slower progression of renal failure following 5/6-nephrectomy [36].

CONCLUSION

Our study demonstrates that it is safe to administer a CDK antagonist for a 4-week period even in instances where podocytes have in part entered the cell cycle and are expressing CDK2 and other CDKs. CYC202 therefore continues to hold promise as a novel therapeutic approach to proliferative glomerular disease.

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